Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms

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ACUTE RENAL FAILURE (ARF) is a syndrome of rapidly declining renal function induced by a number of different insults. In clinical practice, ischemia-reperfusion (I/R) injury is the most common cause for this complication. Although a number of agents and growth factors have been proven effective in the amelioration of ARF in otherwise healthy animals, no significantly effective new therapy has been introduced into clinical practice in decades (10). It is for these reasons that fundamentally new strategies for the treatment of ARF are needed.

Recently, the importance of disordered vascular function and inflammation in the overall pathophysiology of ARF has been increasingly recognized (4, 19). From this would follow that new therapies effectively targeting vascular dysfunction and inflammation should improve the outcome in ARF by providing more favorable intrarenal conditions for tubular self-regeneration and thus kidney repair.

Another therapeutic principle that might at least theoretically improve recovery in ARF is the administration of viable tubular and vascular endothelial cells or their respective precursors, cells that would be capable of homing to the injured kidney, integrate, and physically replace cells that have been destroyed in ARF.

We propose that treatment with pluripotent, adult stem cells offers, compared with pharmacological interventions, a broad therapeutic spectrum through which vascular, inflammatory, and other manifestations of ischemic ARF can be simultaneously targeted. This reasoning is based on the fact that administered stem cells are functionally intact and readily reach intrarenal sites of injury via the circulation. There, they can react physiologically to different local stimuli, for example, hypoxia or ischemia, in turn leading to the release of vasoactive factors, growth factors, immunomodulatory cytokines, and chemokines. To further potentiate the secretion of renoprotective factors by stem cells, we genetically engineered cells to express erythropoietin (38).

In clinical practice, bone marrow-derived stem cells have been most extensively employed in bone marrow transplantation. Their use is facilitated by their ready availability, ease of harvest, and suitability for effective ex vivo enrichment. The bone marrow contains two stem cell types, hematopoietic stem cells (HSC), giving rise to all differentiated blood cells, and mesenchymal stem cells or marrow stromal cells (MSC), which support hematopoiesis in the stem cell niche and differentiate into mesenchymal cells such as chondrocytes, osteocytes, and adipocytes (28).

Stem cells are undifferentiated cells that undergo both self-renewal and differentiation into one or more cell types (37). They can be categorized cytologically, functionally, based on their expression of cell surface markers, transcription factors, and cytokines; however, stem cells are generally rare and difficult to detect.

It has been hypothesized that under physiological steady-state conditions, organ intrinsic stem cells replenish cells lost by normal turnover, and circulating or mobilized stem cells function as back-up units in case the organ intrinsic system is unable to adequately support tissue repair; however, both

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systems can be overwhelmed if tissue damage is more severe, leading to extensive and often permanent organ damage (15). Secretion of regulatory chemokines and cytokines has been recently reported to mediate homing to and transdifferentiation of bone marrow-derived cells in injured organs. This was concluded since bone marrow cells did not contribute to tissue regeneration in the absence of injury (35). Although transdifferentiation of adult stem cells is a recognized phenomenon, controversy still exists regarding its actual existence and frequency (36). In addition, it is also apparent that the effectiveness of stem cell therapy does not exclusively depend on this phenomenon (22). Despite the recent negative reports about transdifferentiation (3, 16, 36), clinical trials with bone marrow-derived cell populations have been published and in part proven effective in the treatment of myocardial infarction (31, 33, 39). Finally, in some injury models fusion of transplanted cells with resident cells was detected instead of actual transdifferentiation (34).

Treatment of human myocardial infarction with stem cells (14, 39) showed that HSC from the bone marrow transdifferentiate into cardiomyocytes and improve organ function as well as survival in a mouse model of myocardial infarction (25). However, these results could not be reproduced by other groups (3, 21). However, such HSC-based protocols have been tested in patients and a modest improvement of cardiac function has been observed, whereas specific mediator mechanisms remain poorly understood (39). Augmented intramyocardial angiogenesis and paracrine effects elicited by administered stem cells are organ-protective mechanisms that are currently favored over transdifferentiation of stem cells into cardiomyocytes (18).

Based on this background, we hypothesized that the already recognized paracrine capabilities of bone marrow-derived mesenchymal stem cells, such as VEGF and HGF secretion (23), and their ability to differentiate into cells of endothelial phenotype (26), may hold particular promise in the treatment of ARF as has been shown by other groups with different cell types (2, 5). In this fashion, the intrarenal delivery of these and other factors and cytokines via administered MSC might prove a potent new technique of targeting vascular dysfunction. This, if found effective, would be expected to reduce the extent of secondary tubular injury and facilitate repair of the ischemically injured kidney (19, 30).

The aim of the present study was, therefore, to test the therapeutic potential of MSC administered either immediately or at 24 h after reflow in a rat model of I/R ARF. In addition, we thought to identify mediator mechanisms that underlie the renoprotective actions of MSC, as well as assess whether infused MSC elicit changes in the renal expression of relevant growth factors, cytokines, and other genes and whether they transdifferentiate into renal cells.

METHODS

Cells and culture. MSC were generated by flushing the femurs of killed rats with sterile PBS and spinning down the cellular content. After resuspension in MEM, cells were filtered through 70-μm mesh (Beckton & Dickinson, San Jose, CA) and plated in 75-cm² primary culture flasks with DMEM/Ham’s F-12 (Sigma, St. Louis, MO) and 10% FCS. Nonadherent cells were removed after 72 h, and adherent cells were passed at low density into new flasks. Cells had a typical spindle-shaped appearance, and the MSC phenotype was confirmed by differentiation into osteocytes and adipocytes with specific differentiation media (28).

Cell labeling for in vivo tracking. Before infusion into control and ARF rats, attached MSC were fluorescence labeled by incubation with 10 μM carboxy-fluorescein diacetate (CFDA; Vybrant cell tracer kit, Molecular Probes, Eugene, OR) in serum-free tissue culture medium (SFM) for 30 min. After trypsinization, labeling was confirmed by fluorescence microscopy and cells were kept on ice in SFM until infusion.

Animals, induction of I/R ARF, and cell infusions. All procedures involving animals were approved by the respective Institutional Animal Care and Use Committees of the University of Utah, Veterans Affairs Medical Center (Salt Lake City, UT), Indiana University (Indianapolis, IN), and the University of Hamburg (Hamburg, Germany). Animals were housed at a constant temperature and humidity, with a 12:12-h light-dark cycle, and had unrestricted access to a standard diet and tap water. For all experiments, groups (n = 6/group) of adult male Sprague-Dawley (SD) and Fisher 344 (F344) rats weighing 200–300 g were used (Charles River, Wilmington, MA).

I/R ARF was induced in isoflurane-anesthetized animals, and rectal temperature was maintained at 37°C. After a midabdominal laparotomy, kidneys were exposed and renal pedicles were clamped withatraumatic vascular clamps for 40 min. While clamps were applied, the left carotid artery was cannulated with PE-50 tubing for intra-aortic cell delivery immediately after reflow. The vehicle in control rats with ARF was infused by the same route. Administration of cells or vehicle was performed either immediately or 24 h after reflow or surgery. Immediately after visual confirmation of reflow, ~10⁶ labeled MSC/animal in 0.2 ml SFM were given via the left carotid artery. Control ARF animals were treated identically but infused with 0.2 ml SFM instead of cells. A separate control group of F344 rats with ARF was infused with ~10⁶ synegetic fibroblasts in 0.2 ml SFM, using an identical protocol. Delayed infusions (~10⁶ labeled MSC in 0.2 ml SFM or 0.2 ml SFM as vehicle) were conducted in isoflurane-anesthetized animals 24 h after reflow via the left carotid artery. Incisions were closed with 4-0 silk, and animals were allowed to recover.

Quantification of cell numbers. CFDA-labeled green fluorescing cells were examined and quantified in kidneys at 2, 24, and 72 h after ARF and cell or vehicle infusions. Nuclei were stained with either Hoechst 33342 or propidium iodide, and nuclei were counted in at least three high-power fields (HPF) per section based on a calibrated confocal micrometer measurement bar. After this, total cell numbers of the studied tissue sections were calculated based on numbers of nuclei and surface area of the section.

Real-time PCR. RNA for real-time PCR was extracted with an RNaseasy kit (Qiagen, Valencia, CA), including a DNase digestion step to exclude contaminating DNA. Reverse transcription was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, CA) for 60 min at 42°C.

Real-time PCR with relative quantification of target gene copy numbers in relation to β-actin transcripts was carried out using the following primers:

<table>
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<th>Gene</th>
<th>Forward primer (5′ → 3′)</th>
<th>Reverse primer (5′ → 3′)</th>
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<td>VEGF-D</td>
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<td>3′ gcagcactgctacaaacaca</td>
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<tr>
<td>HB-EGF</td>
<td>5′ ttccctgcttctgcac 3′</td>
<td>cctgttggtgtgacact</td>
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<td>3′ tgtgctgctcttcttgagag</td>
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infused. A Bio-Rad MRC-1024 two-photon laser-scanning microscope labeled with FITC-labeled albumin, when rhodamine-dextran-labeled MSC were placed in a petri dish filled with normal saline. The renal line was inserted into the femoral artery, and its tip was advanced to the O'Brien Center, Division of Nephrology (directed by Dr. Bruce Molitoris, University of Indiana). Adult male SD rats, weighing 200–300 g, were anesthetized with isoflurane, and ischemic ARF was induced as described above. For delivery of cells, a PE-50 catheter was advanced to the typical melting temperature for a given product. Specificity of the product was determined by generation of a melting curve, and gels were run to control for the formation of unspecific bands. Sample were run in duplicate, and the average crossing point (CP) value was used for calculations. The CP, which is the cycle at which the amount of amplified gene of interest reached a threshold above background fluorescence, was determined to quantify initial starting copy numbers. The relative quantity of mRNA expression was calculated with the comparative CP method using the following formula (27)

\[ \text{ratio} = \left( \frac{E_{\text{target}}}{E_{\text{reference}}} \right)^{\frac{C_{\text{reference}} - C_{\text{target}}}{C_{\text{target}} - C_{\text{reference}}}} \]

where \( E \) is the real-time PCR efficiency, \( CP \) is the crossing point, and \( \Delta \) is the difference of a sample vs. control.

The relative quantitation value of a target gene, normalized to \( \beta \)-actin as the internal control gene, is expressed as a number, which indicates the relative expression compared with that gene. To avoid the possibility of amplifying contaminating DNA and unspecific amplification, the following precautions were taken: 1) a DNase-digestion step was included in the RNA-extraction protocol, 2) some primers were designed to include an intron sequence inside the cDNA to be amplified, 3) reactions were performed with appropriate negative controls (template-free controls), 4) a uniform amplification of the products was rechecked by analyzing the melting curves of the amplified products (dissociation graphs), and 5) gel electrophoresis was performed to confirm both the correct size of the amplification products and the absence of unspecific bands, respectively.

**Y chromosome PCR.** DNA extraction was carried out with the Qiang DNeasy tissue kit. Amplification was performed as above with DNA instead of cDNA. Sensitivity of the Y-chromosome PCR for detection of male cells in a female background was determined by adding initially 10^4 male MSC to 10^6 female leukocytes (10^-2 dilution), followed by stepwise 10-fold dilutions of male MSC down to a number of 1 in 10^5 female leukocytes.

**Homing studies.** In vivo homing studies of administered MSC were performed at the O'Brien Center, Division of Nephrology (directed by Dr. Bruce Molitoris, University of Indiana). Adult male SD rats, weighing 200–300 g, were anesthetized with isoflurane, and ischemic ARF was induced as described above. For delivery of cells, a PE-50 line was inserted into the femoral artery, and its tip was advanced to position in the suprarenal aorta. MSC were labeled either with CFDA as described above or by 24-h incubation with rhodamine-dextran [molecular weight (MW) 10,000, Molecular Probes]. For in vivo microscopy, anesthesia was switched to pentobarbital sodium (50 mg/kg), and the left kidney was exteriorized through a flank incision and placed in a petri dish filled with normal saline. The renal vasculature was either visualized by infusion of large-MW dextran-rhodamine (MW 500,000), in the case of CFDA-labeled MSC, or FITC-labeled albumin, when rhodamine-dextran-labeled MSC were infused. A Bio-Rad MRC-1024 two-photon laser-scanning microscope (Bio-Rad, Hercules, CA) mounted on a Nikon Diaphot inverted stage platform (Fryer, Huntley, IL) with a Ti:sapphire laser (Spectra-Physics, Franklin, MA) was used for in vivo observations. Acquisition variables and placement of the rat on the microscope stage were performed as previously described (12). All images were collected with the use of an ×60 water-immersion objective with a numerical aperture of 1.2. A wavelength of 800 nm was used to excite the mixture of fluorescent probes.

For cell-homing studies at 2 and 24 h after infusion of MSC, animals were killed at these time points and 8-μm snap-frozen kidney sections (OCT, Tissue Tek, EMS, Hatfield, PA) were analyzed by fluorescence microscopy on a deconvolution microscope.

**Histology and injury scores.** We stained coronal sections of fixed kidneys with hematoxylin and eosin and scored the degree of tubular injury by a previously reported approach (6) in random cortical fields using a graticulated grid with 25 squares with an ×20 objective. One member of our team examined 100 intersections between tubular profiles and the grid for each kidney. A score for each tubular cross section per intersection was assigned as follows: 0 = normal histology; 1 = tubular cell swelling, loss of brush border, nuclear condensation (apoptosis), up to one-third of tubular cross section showing nuclear loss (necrosis); 2 = same as for score 1, except for greater than one-third and less than two-thirds of nuclear loss per tubular cross section (necrosis); and 3 = greater than two-thirds of tubular cross section shows nuclear loss (necrosis). The total score per kidney was calculated by addition of all 100 scores with a maximum possible injury score of 300. Leukocyte infiltration per millimeter was scored as reported before (32).

**Immunohistochemistry.** Paraffin sections of kidneys were deparaffinized with xylene and rehydrated in an alcohol series and water. After incubation with a peroxidase-blocking reagent, slides were labeled with a 1:1,000 dilution of a primary anti-fluorescin/Oregon green antibody (Molecular Probes) for 60 min, and CFDA-positive cells were visualized with the EnVision system (Dako, Carpentry, CA). For proliferating cell nuclear antigen (PCNA) staining, a monoclonal mouse anti-rat PC12 antibody was used in a ready-to-use formulation (Dako). Scoring for PCNA-positive cells, a marker of mitogenesis, was carried out by counting the number of positive nuclei in four randomly chosen sections of kidney cortex and outer medulla using ×20 magnification. Data from all fields and all kidneys were pooled to obtain PCNA scores. Apoptotic scores were obtained with a terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Accordingly, kidney sections were deparaffinized, rehydrated, and digested with protein K and labeled with TUNEL reaction mixture for 60 min at 37°C. Sections were screened for positive nuclei under a fluorescence microscope, and 10 random sections in the cortex and outer medullary were counted for every kidney under ×40 magnification. Data from all fields and all kidneys were pooled to obtain apoptotic scores.

**Fluorescence in situ hybridization.** Frozen kidney sections (4 μm) were hybridized with a Starfish rat Y chromosome probe (Cambio, Cambridge, UK). Slides were fixed with methanol:acetic acid (3:1) and dehydrated by serial ethanol washings. After denaturation at 70°C, slides were incubated with the denatured probe at 37°C overnight, and posthybridization three rinses with PBS were performed at 37°C and nuclei were counterstained with 4',6-diamidino-2-phenylindole.

**Cytokine arrays.** Decapsulated kidney tissues were minced, sonicated, lysed with RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor) for cell and tissue lysis, and protein was quantified by BCA protein assay (Pierce, Rockford, IL). The cytokine arrays were performed by RayBiotech according to their protocols for the RayBio Rat Cytokine Antibody Array I (R0608001A, RayBiotech, Norcross, GA). Relative intensities of obtained spots were measured by densitometry and corrected by background subtraction. Results of duplicate readings were averaged.

**Statistical analysis.** Data are expressed as means ± SD. Primary data collection utilized Excel (Microsoft, Redmond, WA), and statis-
tical analyses were carried out using Prism (GraphPad, San Diego, CA). ANOVA and t-tests were used to assess differences between data means as appropriate. A P value of <0.05 was considered significant.

RESULTS

MSC were generated by standard procedures and grown for at least three passages in culture. Contaminating hematopoietic cells were depleted during passaging, and MSC were morphologically defined by a fibroblast-like appearance. Before use, each batch of MSC was further characterized by confirming their specific ability to undergo osteogenic and adipogenic differentiation (Fig. 1). Only cells that met these criteria were used in subsequent experiments.

Administered MSC improve kidney function after I/R ARF. Administration of MSC either immediately or 24 h after reflow significantly improved renal function in animals at days 2 and 3, as assessed by serum creatinine (Fig. 2) and blood urea nitrogen levels (not shown). At 24 h postischemia, renal function was identically decreased in animals designated to receive MSC or vehicle treatment. However, subsequent to MSC administration renal function improved significantly compared with that in vehicle-treated animals (Fig. 2B). The experiments summarized in Fig. 1A were conducted at sea level in Hamburg, Germany, using SD rats and 40 min of bilateral renal pedicle clamping. This resulted in the vehicle-treated control group being in moderate renal failure, with a serum creatinine of 2.3 ± 0.3 mg/dl at 24 h.

We next assessed the effect of MSC, vehicle, or syngeneic fibroblast administration on the early course of ARF in male F344 rats in Salt Lake City, UT (1,400 m above sea level). Apparently, due to the higher altitude or higher relative sus-

Fig. 1. Mesenchymal stem cells (MSC) were characterized by their canonical ability to differentiate into adipocytes and osteocytes.

Fig. 2. Infused MSC (arrows) are significantly renoprotective when administered immediately after (A) or 24 h after reflow (B; Sprague-Dawley rats). Open bars, vehicle-treated animals; filled bars, MSC-infused animals. P, statistical difference between serum creatinine (Scr) in vehicle- vs. MSC-treated animals.
ceptibility of male F344 rats (unpublished observations), 40 min of clamping in these animals led to more severe renal insufficiency, as evidenced by a rise in serum creatinine to $4.5 \pm 0.5$ mg/dl in vehicle-treated rats at 24 h after cell injection ($P = 0.002$) compared with both vehicle- or fibroblast-treated animals (Fig. 3A). Rats were killed at 24 h for scoring of renal injury, leukocyte infiltration, PCNA, and TUNEL staining, respectively. Compared with control kidneys, kidneys from MSC-treated rats had significantly lower injury scores ($P = 0.004$, Fig. 3B), similar leukocyte infiltration scores (not shown), a significantly higher PCNA staining index ($P = 0.023$, Fig. 3C), and reduced numbers of apoptotic cells on TUNEL assay ($P < 0.0001$, Fig. 3D).

Administered MSC are detected in the kidney by in vivo microscopy. Infused MSC were tracked in ARF and control kidneys in vivo, using two-photon laser-confocal microscopy at the O’Brien Center (Indianapolis, IN). MSC were prelabeled either with tetramethylrhodamine dextran, MW 10,000, robustly taken up by endocytosis and emitting a red fluorescence signal, or CFDA, emitting a green fluorescence signal; renal vessels then were either visualized by infusion of high-molecular-weight FITC-dextran, MW 500,000, emitting a green fluorescence signal, or albumin-rhodamine, emitting a red fluorescence signal. Either combination of fluorophores facilitates a red-green combination with excellent color discrimination between labeled cells and intravascular markers, respectively. Although observation depth with this microscope is limited to $\sim 100 \mu$m, infused, free-flowing cells were detected in the renal microvasculature in ARF and control animals immediately after administration, and occasional cells remained firmly attached in an intravascular, subcapsular, and occasional peritubular capillary location over the 30 min of observation. In addition, a rare superficial glomerulus was found to contain one or more MSC (Fig. 4).

Administered MSC are detected histologically in the damaged kidney and other organs. To further evaluate the renal delivery of intra-arterially infused, CFDA-labeled MSC, F344 rats with I/R ARF were killed 2, 24, and 72 h after cell administration, and blood and organs were examined for the presence of MSC by fluorescence microscopy and immunostaining. At all study times, peripheral venous blood did not contain infused cells, indicating that they were rapidly cleared from the circulation. However, administered cells were detected by immunofluorescence microscopy in the kidney, liver, lungs, bone marrow, and spleen at 2 h after infusion. Heart tissue did not contain labeled cells at this time point. Almost all MSC in the kidney were located inside glomerular capillaries (Fig. 5). By 24 h, occasional cells were still detected in the lung, whereas no cells were found in the kidney. At 72 h after cell infusion, no cells could be detected in the kidney by fluorescence microscopy.

To verify fluorescence results and to increase detection sensitivity, we stained kidney sections of animals infused with CFDA-labeled cells with an antibody against CFDA and visualized cells with the DAKO envision kit. On confocal microscopy, renal sections from animals killed at 2 h after cell injection showed MSC primarily in glomerular capillaries (Fig. 4).
Fig. 4. In vivo tracking of infused MSC by 2-photon laser confocal microscopy. A and B: green-labeled MSC (arrows) attached in peritubular capillaries within ~10 min of intra-arterial injection. Plasma is visualized in red with rhodamine-albumin, and kidney tubules show a granular, yellowish autofluorescence. C: MSC, red labeled with rhodamine-dextran, are attached in a peritubular capillary (arrow), and capillaries are visualized with green FITC-dextran. D: at 24 h post-reflow, single MSC (arrows) are detected in a glomerulus (MSC stained red with rhodamine-dextran, and nuclei stained blue with Hoechst 33342).

Fig. 5. Two hours after infusion and reflow, MSC are detected in glomeruli. A–C: glomeruli (dashed circles) with and without green-labeled MSC. Approximately 60% of glomeruli (nuclei stained red with propidium iodide) contained 2–10 labeled MSC [green fluorescent carboxy-fluorescein diacetate (CFDA)]. B: 3 glomeruli in the outer cortex, 2 of them containing green-labeled MSC. D: immunohistochemistry for CFDA confirms the glomerular location of infused MSC (arrow), and the specificity of the fluorescence images in A–C. Magnification: ×40 (A) and ×20 (B and C) using confocal microscopy; ×20 (D).
sought to track administered MSC with a stable genetic marker. Therefore, female rats with ARF were infused with male CFDA-labeled cells, and the cells were visualized, in addition, with Y chromosome fluorescence in situ hybridization (FISH). FISH for the Y chromosome had a sensitivity of \(-65\%\) (Fig. 6), corresponding to published reports (29). We did not detect any Y chromosome- and CFDA-positive cells at 24 h in the kidneys of MSC-infused animals, thereby corroborating the negative results above.

To confirm the FISH data, we next performed Y chromosome PCR. The sensitivity of this method was \(10^{-4}\) or 100 cells detected in a total of \(10^6\) cells that were screened (Fig. 7). Organs of female rats infused with male MSC were harvested at 24 h and at day 3 after infusion. Real-time quantitative PCR with Y chromosome-specific primers showed amplification only in the lungs at 24 h after cell infusion, whereas kidney, liver, spleen, and bone marrow were negative, thereby further corroborating our results above (Fig. 7).

**Gene expression in MSC-infused kidneys.** Due to the unexpectedly low numbers of infused cells detected in the kidney at 24 h and later, i.e., at a time when significant renoprotection was clearly established, we sought to further elucidate potential mechanisms whereby administered MSC exert their protective effects. Because MSC are known to secrete growth factors and have immunomodulatory properties, we screened kidneys, by real-time quantitative RT-PCR, for changes in the expression of growth factor-, inflammatory-, apoptosis-related and nitric oxide synthase (NOS) genes (Fig. 8). Kidneys of animals treated with MSC showed at 24 h post-cell administration a highly significant reduction in the expression of genes encoding the proinflammatory cytokines IL-1\(\beta\), TNF-\(\alpha\), and IFN-\(\gamma\), as well as inducible NOS (iNOS), whereas the anti-inflammatory cytokine IL-10 was robustly expressed in MSC-treated and not in vehicle-infused animals (Fig. 8A). The renal expression of VEGF-A, -B, -C, and -D, EGF, HB-EGF, IGF-I, and

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**Fig. 6.** Y chromosome FISH of a kidney section from a male Fisher 344 rat. Approximately 65\% of the nuclei in this 4-\(\mu\)m section are Y chromosome positive (red), examined by deconvolution microscopy; nuclei were stained with 4',6'-diamidino-2-phenylindole. Magnification: \(\times40\).

**Fig. 7.** Sensitivity of Y chromosome PCR assessed by serial dilution. A: PCR for male MSC diluted in female leukocytes is positive up to a dilution of \(10^{-4}\). Dilution steps are marked by \(-2\) to \(-6\), indicating a \(10^{-2}\) to \(10^{-6}\) dilution, respectively. Lane 1, DNA size markers. NTC, nontemplate control. B: Y chromosome PCR at 24 h after MSC infusion from a male donor into female recipients with ARF. The only organ positive for Y chromosome DNA was the lung (lane 2). Kidney cortex and medulla as well as liver and spleen from 2 animals were negative (lanes 3–11). Lane A, female DNA; lane B, male DNA; lane 2, lung; lane 3, kidney cortex; lane 4, kidney medulla; lane 5, liver; lane 6, spleen; lane 7, wound scar; lane 8, lung; lane 9, bone marrow; lane 10, liver; lane 11, spleen. The gel was run following amplification with the Cepheid real-time PCR SmartCycler.
Fig. 8. Comparative gene expression ratios in ARF kidneys of MSC- and vehicle-treated animals, generated by referencing each gene to β-actin as internal control. A: MSC treatment caused significant \( (P < 0.05) \) suppression (>10-fold) of proinflammatory IL-1β, TNF-α, and IFN-γ (nos. above bars indicate actual values). Anti-inflammatory IL-10 was robustly expressed in MSC-, shown as an arbitrary value, and not in vehicle-treated animals. Filled bars on all panels depict gene expression ratio of 1, i.e., a value obtained when gene expression ratios between MSC- and vehicle-treated animals are “equal.” Values above or below 1 indicate that gene expression in MSC-treated animals has increased or decreased compared with vehicle-treated animals. B: MSC treatment caused increased gene expression of bFGF and TGF-α, whereas that of HGF was suppressed. C: antiapoptotic Bcl-2 expression was robustly induced, whereas that of inducible nitric oxide synthase (iNOS) was suppressed in MSC- vs. vehicle-treated animals. eNOS, endothelial NOS.
BMP-7 was essentially comparable in MSC- and vehicle-treated animals. On the other hand, MSC-treated animals showed a 10-fold reduction in HGF expression, whereas that of bFGF increased 2.8-fold and that of TGF-α 8-fold (Fig. 8B). The antiapoptotic Bcl-2 gene was only expressed in MSC-treated but not in control animals, whereas there was no significant difference in Bcl-XL, Bcl-XS, NF-κB, and endothelial NOS expression between MSC- and vehicle-treated animals.

Gene expression in administered MSC and fibroblasts. As shown in Fig. 2A, MSC infusion led to significant and prompt renoprotection, whereas administration of fibroblasts or vehicle had no such effect. We compared, therefore, the expression of growth factor genes in MSC to that in fibroblasts (Fig. 9). MSC exhibited significantly higher expression of IGF-I, which was not expressed in fibroblasts, and higher expression of HGF, VEGF-B, and VEGF-D. In contrast, the expression in MSC of EGF, HB-EGF, BMP-7, and bFGF were lower than that in fibroblasts.

Cytokine arrays. To test whether renal cytokine expression profiles determined by PCR, as shown in Fig. 7, correspond with actual tissue protein levels, we performed semiquantitative protein assays with a cytokine array system (Fig. 10). IFN-γ and IL-1β levels were lower in treated animals corresponding with PCR results, whereas levels of IL-10 were similar, and those of TNF-α and VEGF were higher in MSC-compared with vehicle-treated ARF animals (Fig. 10A). However, due to the large variance in the duplicate densitometric data, none of the averaged results comparing MSC- and vehicle-treated animals reached statistical significance. In addition, MSC treatment caused lower intrarenal protein levels of GM-CSF, IL-1α, leptin, MIP-3, and NGF-β, whereas a series of other cytokine levels remained unchanged (Fig. 10B). The lack of correlation between the results obtained by PCR and cytokine array for IL-10, TNF-α, and VEGF is likely due to different degradation times for the respective mRNAs and their translation products, respectively.

**DISCUSSION**

The present study provides the first clear evidence that therapy with MSC affords significant renoprotection in rats with I/R ARF. Animals infused with MSC either immediately or 24 h after reperfusion had significantly better renal function, lower renal injury and apoptotic scores, and higher mitogenic indices than vehicle-treated animals. This was demonstrated in two different rat strains with moderate or more severe degrees of ARF, respectively. In contrast, the administration of equal numbers of syngeneic fibroblasts had neither renoprotective nor adverse effects. Using in vivo and in vitro techniques, infused MSC were detected in the kidney only early after administration and were predominantly in glomeruli and attached in peritubular capillaries. After 24 h, not any or only exceptionally scarce numbers of MSCs were found in the kidney, a pattern that essentially rules out the possibility that significant numbers of infused MSC are retained in the kidney where they could physically replace lost kidney cells by transdifferentiation. This conclusion is furthermore supported by the fact that there were no intrarenal transdifferentiation events of MSC within 3 days of administration, whereas occasional MSC-derived capillary endothelial cells were identified only after 5–7 days (not shown). From this, we deduce that the mechanisms that mediate the protective effects of MSC must be primarily paracrine, as implied by their expression of several growth factors such as HGF, VEGF, and IGF-I, all known to improve renal function in ARF, mediated by their antiapoptotic, mitogenic and other cytokine actions (24, 41). Collectively, these as yet incompletely defined paracrine actions of MSC result in the renal downregulation of proinflammatory cytokines IL-1β, TNF-α, and IFN-γ, as well as iNOS, and upregulation of anti-inflammatory and organ-protective IL-10 (8), as well as bFGF, TGF-α, and Bcl-2. The lack of renoprotection obtained by infused fibroblasts may be due, at least in part, to the fact that MSC exhibit a comparatively higher expression of VEGF, HGF, and IGF-I, therefore suggesting that the combined delivery, by MSC, of these factors appears to result in superior renoprotection than that obtained with the growth factors that are more highly expressed by fibroblasts (EGF, HB-EGF, BMP-7, bFGF).

The current studies were conducted in rodents with I/R ARF, an extensively investigated, albeit imperfect model of the most common and the most treatment-resistant type of clinical ARF (17). In this model, when the injury is of modest intensity, the decrease in renal function immediately following I/R injury is maximal at 24–48 h, followed within several days by almost complete normalization of renal function. Morigi et al. (20), using rodents with cisplatinum-induced ARF, showed that administration of MSC improved renal function, and MSC appeared to directly contribute to the reconstitution of renal function.

![Comparison of growth factor expression in MSC and fibroblasts](image-url)
epithelium by transdifferentiation. However, these investigators did not demonstrate that the observed transdifferentiation of MSC is the actual mechanism of renoprotection, and they presented no data regarding the actual numbers of donor cells that undertook the tubular repair.

There has been much debate about transdifferentiation of bone marrow-derived cells and a number of contradicting reports have been published showing either “transdifferentiation” or not (3, 25). It may be important, in this context, that the kinetics of the cisplatinum model are fundamentally different from those of the I/R model. Specifically, in this nephrotoxic model maximal injury occurs on day 7 after cisplatinum application, which, at least in theory, provides the earlier infused MSC significantly more time to transdifferentiate into renal target cells. In contrast, we obtained significant functional improvement in severe ARF as early as 24 h following reflow and infusion of MSC, i.e., at a time point that would be too early, as we show here, for tubular replacement to occur via transdifferentiation of administered stem cells.

Intravenous or intra-arterial infusion of MSC results in entrapment of administered cells in capillary beds of most organs, but most prominently in the lung, and also in the liver as late as 48 h postinfusion (13). We were, however, unable to reproduce the latter observation. This entrapment of MSC likely occurs because of the relatively large size of these cells, averaging ~20–30 μm in diameter. Overall, in normal animals, administered MSC have not been found to remain detectable beyond a few days in organs other than the bone marrow (9).

Having documented in the present study the rather early therapeutic efficiency of MSC in ARF, which makes direct, physical replacement of damaged resident cells by donor cells unlikely, we next investigated alternative, differentiation-independent mediator mechanisms that could explain the renoprotective effects of these cells. MSC are known to have incompletely understood immunomodulatory properties that result in the inhibition or modulation of the T cell response, and they secrete various growth factors and cytokines (1, 7). T cell responses are likely involved in the pathogenesis of ARF, and their modulation by MSC might be a possible mechanism of protection, i.e., analogous to the observations made by Yokoto et al. (40). We observed significant differences in cytokine and growth factor expression in MSC-treated kidneys, likely the direct or secondary result, via primary improvement of kidney injury, of this therapy. Expression of proinflammatory cytokines IL-1β, TNF-α, IFN-γ, as well as iNOS, was reduced, whereas anti-inflammatory IL-10 was upregulated. Expression of known renoprotective growth factors bFGF, TGF-α, and antiapoptotic Bcl-2 was increased in MSC-treated kidneys, whereas HGF, highly expressed in MSC, was surprisingly...
downregulated. Infused fibroblasts, as we show, express high levels of a different spectrum of known renoprotective growth factors, such as EGF, HB-EGF, BMP-7, and bFGF. However, they lacked therapeutic efficiency in ARF, possible due to these differences in the types and mix of growth factors expressed by MSC and fibroblasts, respectively. As already pointed out above, an additional explanation for the therapeutic superiority of MSC may be their immunomodulatory capacity that fibroblasts lack (1). Finally, in contrast to whole kidney changes, relatively little is known to date about microenvironmental modifications in growth factor and cytokine expression and their secretion by sublethally injured or stressed resident cells in I/R ARF. In addition, it is furthermore likely that milieu changes at sites of injury induce the synthesis and secretion of additional factors that are currently unknown or not readily assayable.

The tracking of administered MSC in the kidney is critical to the interpretation of our experimental results. It has been argued that genetic markers like the Y chromosome or eGFP are superior to nongenetic tracking methods. However, the utility of eGFP as a cell marker is also limited because the kidney possesses intensive autofluorescence, which makes it difficult to detect eGFP-positive MSC unless confocal microscopy is used. β-Gal, another cell marker, has been shown to yield false positive results when pH is not strictly controlled during histological processing (11). In the present studies, we avoided inadequate sensitivity of a single-cell tracking method by utilizing both genetic and nongenetic cell-tagging techniques. We found that there was complete agreement in the data obtained with either approach, indicating that combining different labeling techniques and molecular assays achieves maximal sensitivity and highest possible specificity for tracking of MSC in the kidney. Although we did not detect transdifferentiation events during the 72-h period of observation, it is possible that cell transdifferentiation and integration may be important at later stages of organ repair. Additional studies are needed to further validate the individual or combined importance of paracrine and transdifferentiation mechanisms in ARF.

The primary advantage of MSC for utilization in cell therapy is the ease with which they can be harvested from the bone marrow, isolated by plastic adherence, expanded in culture, genetically engineered, differentiated, and handled in vitro (28). However, in vitro manipulations may also alter or influence their natural phenotype, leading to different, as yet undefined activities and responses. To control for this possibility, we regularly tested the canonical ability of cultured MSC to undergo lipogenic and osteogenic differentiation; i.e., we confirmed with this approach that subsequently administered cells had retained the characteristics of MSC.

There are currently no reports showing adverse effects of adult stem cells used in cell therapy. Although this suggests a great advantage over embryonic stem cells, which have been documented to give rise to teratomas, long-term studies will have to be conducted to prove that no adverse effects occur after in vivo administration of adult MSC.

In summary, our present studies clearly demonstrate that administration of MSC to animals with I/R ARF is highly renoprotective and that these beneficial effects are predominantly mediated, as our data suggest, by paracrine rather than transdifferentiation-dependent mechanisms. Our observations are furthermore compatible with the notion that the potentially unique mix of growth factors elaborated by MSC may explain their significant renoprotective activity that is not obtained with fibroblasts, cells that express a different growth factor spectrum. The collective and individual renoprotective capacity of cytokines temporarily released by MSC is currently undergoing investigation. It is surprising that the very transient presence of MSC in the injured kidney, as we document, is sufficient to greatly ameliorate the course of I/R ARF. Protective and repair mechanisms that are activated by MSC resemble those that can be induced by individual growth factors in experimental ARF. We documented antiapoptotic, mitogenic, and anti-inflammatory responses, evidenced by both improved tissue scores and changes in the expression of mechanism-specific genes. Whether the renoprotective and gene-modulating effects of MSC are primary actions that are humorally elicited by these cells or whether they result from the improvement of tissue injury by as yet unknown factors released by them remains to be determined. Future studies will also have to define the possible contribution to organ protection made by the immunomodulatory effects of MSC.

In conclusion, we believe that successful treatment of I/R ARF with MSC demonstrated herein holds substantial promise for the development of novel, MSC-based interventions that can improve the treatment of severe, and still largely therapy-resistant, clinical ARF that results from I/R injury. Pluripotent MSC, because of their versatility and the ease with which they can be harvested from the bone marrow, culture expanded, and engineered, appear to be a particularly well-suited stem cell type for these clinical indications.

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